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The role of nitrite and nitric oxide under low oxygen conditions in plants

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Summary

Plant tissues, particularly roots, can be subjected to periods of hypoxia due to environmental circumstances. Plants have developed various adaptations in response to hypoxic stress and these have been extensively described. Less well-appreciated is the body of evidence demonstrating that scavenging of nitric oxide (NO) and the reduction of nitrate/nitrite regulate important mechanisms that contribute to tolerance to hypoxia. Whilst ethylene controls hyponasty and aerenchyma formation, NO production apparently regulates hypoxic ethylene biosynthesis. In the hypoxic mitochondrion, cytochrome c oxidase, which is a major source of NO, is also inhibited by NO, thereby reducing the respiratory rate and enhancing local oxygen concentrations. Nitrite can maintain ATP generation under hypoxia by coupling its reduction to the translocation of protons from the inner side of mitochondria and generating an electrochemical gradient. This reaction can be further coupled to a reaction whereby non-symbiotic haemoglobin oxidizes NO to nitrate. In addition to these functions, nitrite has been reported to influence mitochondrial structure and supercomplex formation, as well as playing a role in oxygen sensing via the N-end rule pathway. These studies establish that nitrite and NO perform multiple functions during plant hypoxia and suggest that further research into the underlying mechanisms is warranted.

Keywords: cytochrome c oxidase, hyponasty, hypoxia, mitochondria, nitric oxide (NO), nitrite.

Introduction

Molecular oxygen facilitates the efficient production of ATP in all aerobic eukaryotic organisms by providing the terminal electron acceptor for the mitochondrial electron transport chain. Oxygen deprivation, leading to a state of hypoxia (low oxygen) or anoxia (no oxygen), compromises the process of oxidative phosphorylation. For plants, this problem typically arises during flooding (Bailey-Serres *et al.*, 2012) as a result of the 10^4-fold reduction in gaseous diffusion in flood water (Armstrong, 1979). Moreover, even under optimal growth conditions, certain dense tissues such as seeds (Borisjuk *et al.*, 2007) and tubers (Geigenberger *et al.*, 2000) are hypoxic, with O_2 concentrations in the range 1 - 50 µM. While the latter observations indicate that plants can cope with low levels of O_2 during normal development, hypoxia inevitably restricts the availability of oxygen for oxidative phosphorylation and increases the importance of fermentation as a source of ATP (Ricard *et al.,* 1994). As a result, plant metabolism must adapt to lower ATP production, including the induction of energy-conserving pathways of sucrose degradation that lead to improved plant performance under hypoxia (Geigenberger *et al.*, 2000; Bologa *et al.*, 2003).

As well as metabolic adaptions to hypoxia, plants have also developed anatomical and morphological adaptations, including the formation of aerenchyma, aerial adventitious roots, and leaf gas films (Bailey-Serres *et al.*, 2012). Responses to hypoxia have been extensively studied in plants and many transcriptional, post-translational and metabolic events that regulate these responses have been identified (Geigenberger *et al.*, 2000; Licausi *et al.*, 2011; Narsai *et al.*, 2017; Fukao *et al.*, 2019). One emerging theme is the involvement of reactive oxygen species (ROS) and nitric oxide (NO) signalling under low oxygen in plants (Pucciariello and Perata, 2017), and in this update the intention is to focus on the multiple roles of NO and nitrite in the hypoxic response.

Hypoxic synthesis and turnover of NO

NO is a free radical signalling molecule that is produced by several oxidative and reductive pathways (Gupta *et al.,* 2011; Astier *et al.*, 2017). The reductive pathways are active under hypoxic conditions, with mitochondria playing a major role in NO production through the action of cytochrome oxidase (COX) and other deoxyhemeproteins (Figure 1). Isolated plant mitochondria typically produce NO at a rate of 1-20 nmol.mg protein^{-1}.h^{-1} within a few minutes of adding NADH and nitrite to a hypoxic incubation medium, and the K_i for oxygen, which inhibits the process, is 0.05% (Gupta *et al.,* 2005). High levels of NO lead to cell death (Wang *et al.* 2013), so if NO is to have other signalling functions under hypoxia it is necessary to have mechanisms for preventing its excessive accumulation.

NO production is countered by NO degradation, with several haem proteins such as flavohaemoglobin, haemoglobin (Hb), myoglobin and their associated reductases fulfilling this role in animal cells (Gardner, 2005) and flavoglobin scavenging NO in yeast (Liu *et al.*, 2000; Cassanova *et al.*, 2005). In plants, Class 1 phytoglobins (Pgb) are efficient NO scavengers (Hebelstrup *et al.*, 2008) and their very high affinity for oxygen (K_m ~ 2 nM) allows them to function under hypoxia (Figure 1). Overexpression of the Pgb gene in barley decreased NO release under hypoxia, while knockdown of the gene increased it, confirming that Pgb makes a significant contribution to the regulation of NO levels (Cochrane *et al.*, 2017). The inhibition of COX by NO (Brown and Cooper, 1994; Cleeter *et al.*, 1994) also facilitates the operation of the oxygen-requiring Pgb-NO cycle under hypoxia by inhibiting respiration at low oxygen concentrations. Thus under hypoxia oxygenated Pgb converts NO to nitrate, and the resulting metphytoglobin is converted back to Pgb by monodehydroascorbate reductase-mediated ascorbate reduction (Igamberdiev *et al.*, 2006; Gupta and Igamberdiev, 2011). The Pgb-NO cycle also regenerates NAD^+ and may be considered an alternative to the usual pathways of fermentation (Igamberdiev and Hill, 2004), although like lactate fermentation, the Pgb-NO cycle is acidifying (Libourel *et al.*, 2006) and thus a potential contributor to acidosis under hypoxia.

S-nitrosoglutathione reductase (GSNOR) is another enzyme that contributes to the regulation of NO levels in plants (Leterrier *et al.*, 2011). GSNOR converts the NO derivative *S*-nitrosoglutathione (GSNO) to oxidised glutathione (GSSG) and ammonia, and it was recently shown that the inhibitory NO-dependent *S*-nitrosation of GSNOR1 (Frungillo *et al.*, 2014) leads to degradation of the enzyme by selective autophagy (Zhan *et al.*, 2018). Elimination of the *S*-nitrosation site in GSNOR abolished the positive effect of NO on the hypoxic germination of Arabidopsis seeds, indicating that the NO-dependent post-translational modification of GSNOR is a physiologically relevant process that contributes to the hypoxic response (Zhan *et al.*, 2018). Elevated GSNO was shown to increase the expression of both alcohol dehydrogenase and pyruvate decarboxylase in germinating Arabidopsis seeds (Zhan *et al.*, 2018) emphasising the importance of GSNOR regulation by NO under hypoxia.

NDB-type dehydrogenases also play a role in NO degradation by forming superoxide anions that convert NO to peroxynitrite ($ONOO^-$) (de Oliveira *et al.*, 2008). This route of NO degradation is stimulated by calcium, and abolished by superoxide dismutase and complete anoxia. These observations indicate that NDB dehydrogenases actively generate superoxide, and are involved in superoxide-dependent NO degradation. NDB-type dehydrogenases were also found to be induced in transgenic Arabidopsis plants with downregulated expression of GSNOR (Frungillo *et al.*, 2013), providing further correlative evidence for their role in NO homeostasis.

The net result of these biosynthetic and degradative pathways is a marked increase in NO production in response to hypoxia. This increase is in turn increasingly implicated in a range of adaptive responses to oxygen deprivation, including hyponasty, aerenchyma formation, oxygen homeostasis, mitochondrial activity and oxygen sensing

Role of NO in hyponasty under hypoxia

The hyponastic response is a highly effective escape strategy employed by most plant species following submergence, shade or elevated ambient temperatures. It is accompanied by a strong directional growth mediated by reversible turgor reactions and changes in the osmotic state of the cells. It mainly depends on the unequal growth rates of two anatomically different sides of the organ in question. Hyponasty can be defined as a type of asymmetric growth, whereby abaxial tissue displays a higher growth rate than the adaxial cells. It is a common feature in leaf blades and petioles of many monocots and dicots (Polko *et al.*, 2011).

During flooding, ethylene plays a major role in the hyponastic response due to its reduced diffusion in submerged tissues (Voesenek *et al.*, 1993). Vreeburg *et al.* (2005) showed that ethylene-mediated hyponastic signaling is characterized by acidification of the apoplast and a higher expression of expansin proteins, both of which play important roles in modifying cell wall structure. Ultimately, ethylene responsive factors (ERFs) mediate hyponasty, however, the function of these proteins is gibberellin (GA) dependent (Polko *et al.*, 2011). Other hormones are also implicated in the process, with auxins such as indole-3-acetic acid (IAA) positively regulating stage-specific submergence-induced hyponasty, whilst abscisic acid (ABA) acts as a negative regulator of this process (Cox *et al.*, 2006).

NO is also involved in the complex signalling network leading to hyponasty. Arabidopsis seedlings produce increased ethylene and NO under hypoxia, and Pgb gene expression (*AtGLB1*) correlated with hyponastic growth (Hebelstrup *et al.,* 2012). These observations led to the proposal that NO likely acts as a regulator of hyponasty via induction of ethylene synthesis. Plants also experience shading and reduced light levels during flooding, and since shading alone can induce an ethylene-dependent hyponastic response (Pierik *et al.,* 2009) it is possible that shade also has an impact on NO metabolism. It is known that light-dark dynamics can influence both NO and nitrite levels (Planchet *et al.*, 2005), but the relevance of this observation under more natural conditions remains to be evaluated.

Role of NO in the formation of ethylene-induced aerenchyma

Another adaptive response of plants to hypoxia is the formation of aerenchyma, the gas-filled tissue that allows the exchange of gases between shoot and root under conditions of flooding and waterlogging (Drew *et al.,* 2000). Schizogenous aerenchyma is formed by a process of cell separation at the middle lamella during cell development, whilst lysigenous aerenchyma is formed as a consequence of the random death of cortical cells. It was previously shown that hypoxia is an inducer of aerenchyma formation (Drew *et al.,* 2000), and that ethylene plays a role in cortical cell death (Yamauchi *et al.* 2014). NO also plays a role in programed cell death (Delledonne *et al.,* 1998; Chen *et al.*, 2009; Wang *et al.*, 2013), and so the possibility arises that NO could play a role in aerenchyma formation in hypoxic tissues given that NO production takes place within a few minutes of the onset of hypoxia (Gupta *et al.*, 2005).

Recently Wany *et al.* (2017) investigated whether ethylene-induced aerenchyma formation in wheat roots required hypoxia-induced NO. Wheat roots produced NO under hypoxia as expected, and scavenging of NO by 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) led to a marked reduction in aerenchyma formation following 24 or 48 hours of hypoxia. Interestingly, it was found that hypoxically-induced NO was important for the induction of the genes encoding 1-aminocyclopropane-1-carboxylic acid (ACC) synthase and ACC oxidase, both of which are required for ethylene biosynthesis, as well as the ethylene-responsive genes *ERF1* and *PDF13*. Cell death events such as increased electrolyte leakage, increased cellulase activity, DNA fragmentation, and cytoplasmic streaming were all inhibited under hypoxia in the presence of the NO scavenger, reinforcing the conclusion that NO is essential for the development of ethylene-induced aerenchyma (Wany *et al.*, 2017). Moreover, ethylene inhibitors and NO scavengers, either alone or in combination, suppressed the genes involved in signal transduction leading to aerenchyma development. These findings suggest that NO plays a role in aerenchyma formation in wheat, acting either upstream of ethylene, or in parallel with it. The involvement of NO in aerenchyma formation was also indicated by the observation that the *respiratory burst oxidative homolog/NADPH oxidase*

(RBOH/NOX) gene, which is known to have a role in aerenchyma formation (Yun *et al.,* 2011), is also induced by NO (Wany *et al.,* 2017). *RBOH/NOX* plays a role in superoxide production, hence the induction of *RBOH/NOX* by NO, correlated with the generation of ROS in wheat cortical cells. Since superoxide reacts with NO to form peroxynitrite, a strong nitrating agent, increased tyrosine nitration was observed during aerenchyma formation. It will be interesting to see whether these results can be replicated under conditions more akin to natural flooding events, since ethylene is known to accumulate to saturating levels rapidly and the possibility of induction by NO has not been considered hitherto (Sasidharan *et al.*, 2018).

Plants are well equipped with detoxification systems to counter the deleterious effects of ROS and NO. In particular, there are many regulatory mechanisms that influence the levels of ROS and NO, thus protecting plants from severe damage under stress conditions. However, during processes such as programmed cell death and aerenchyma formation, plant cells need to maintain high ROS levels in the tissues where limited cell death is advantageous. One strategy to achieve this is to lower antioxidant gene expression (Liu *et al.*, 2018). Wany and Gupta (2018) found an inverse correlation between antioxidant gene expression and increased ROS following 24 h of hypoxia in wheat roots during aerenchyma formation. NO is known to increase antioxidant gene expression during stress (Tossi *et al.*, 2011), but during cell death it seems that suppression of the antioxidant mechanism is required. A prominent example is the inhibition of glycine decarboxylase by *S*-nitrosation, which alters cellular redox status and promotes cell death (Palmieri *et al.*, 2010). A survey of *S*-nitrosylated proteins in cells undergoing aerenchyma formation could potentially afford new insights into the role of NO in the promotion or limitation of cell progression. Such insights could be based on transgenic manipulation of root NO levels, for example by over-expression of Pgb, during aerenchyma formation, although this approach might also affect oxygen homeostasis and root development (Gupta *et al.,* 2014). An alternative approach might be to test the relationship between the nitrogen status of the soil and aerenchyma formation, given that the synthesis of NO ultimately depends on the availability of nitrate (Planchet *et al.*, 2005; Gupta *et al.,* 2013). Preliminary evidence suggests that nitrate nutrition, as

opposed to ammonium nutrition, favours aerenchyma formation and this needs further investigation (Wany and Gupta, 2018).

While the recent evidence suggests that NO is essential for the development of aerenchyma via cortical cell death under flooding stress (Wany *et al.*, 2017), it is necessary to regulate the levels of NO in other root zones to avoid cell death in tissues required for continued growth (Mira *et al.*, 2016a). The root apical meristem (RAM) contains stem cells which are important for root growth and it has been shown that transgenic suppression of the hypoxically-induced phytoglobins ZmPgb1.1 or ZmPgb1.2. led to structural abnormalities in RAM (Mira *et al.*, 2016b). Suppression of Pgb also enhanced expression of ethylene biosynthetic and responsive genes, providing further support for the role of Pgbs in regulating NO levels under hypoxia. In contrast, overexpression of Pgb improved hypoxic root growth by alleviating apical meristem cell death again emphasising the role of Pgbs as NO scavengers. These observations highlight the need for differential regulation of Pgb expression in different root zones to ensure that hypoxically-induced NO can promote cortical cell death and aerenchyma formation without damage to the stem cells in the RAM. The mechanism by which this is achieved is not fully understood, but it is relevant that exposure of the root apex to hypoxia has been shown to lead to increased hypoxic acclimation of the entire root (Mugnai *et al.*, 2012), emphasising the existence of systemic signalling pathways that coordinate cell-type specific responses to hypoxia.

Role of NO in oxygen homeostasis

Oxygen homeostasis is important for maintaining an appropriate internal oxygen level in tissues during normal development. This phenomenon is crucial when environmental effects, such as flooding and waterlogging, reduce the oxygen supply and drive the tissues towards anoxia. Recently it was shown that NO has a potential role in oxygen homeostasis under normoxia via the regulation of respiration (Gupta *et al.,* 2014). It is well known that NO inhibits respiration by inhibiting cytochrome c oxidase in isolated mitochondria (Millar and Day, 1998). NO binds to the Fe^{2+}-haem

group at the O_2-binding site of the binuclear centre $Fe_{a3}Cu_B$ in COX (Cleeter *et al.,* 1994) and this provides the basis for an autoregulatory mechanism in which increasing NO under hypoxia reduces oxygen consumption. The relevance of this for oxygen homeostasis has been demonstrated in normoxic barley roots, where overexpression of Pgb promoted NO scavenging, increased the respiration rate, and decreased the internal oxygen level (Gupta *et al.*, 2014). Overexpression of Pbg also affected the normoxic NO signalling pathways in barley (Cochrane *et al.,* 2017). The physiological significance of this effect has been shown in both seeds (Borisjuk *et al.*, 2007) and isolated mitochondria (Benemar *et al.*, 2008), where it was shown that nitrite reduction at complex III reversibly inhibited COX, and thus contributed to the maintenance of a steady state level of oxygen in the mitochondria.

The role of NO in oxygen homeostasis is also important in seed germination. This process is associated with the production of NO and a decrease in ABA via regulation of *CYP707A2* transcription and (+)-abscisic acid 8′-hydroxylase (Liu *et al*., 2009). Gibbs *et al.* (2014b) reported that both NO and oxygen availability promote degradation of ERF VII transcription factors during the metabolically active state of seed development, leading to down-regulation of AB15 in the endosperm and the promotion of germination.

Role of NO in mitochondrial activity under hypoxia

Oxygen deprivation can have a marked effect on plant mitochondrial structure, and the observed changes correlate to some extent with the ability of the plant to survive periods of hypoxia or anoxia (Vartapetian *et al.*, 2003; Shingaki-Wells *et al.*, 2014). Nitrate has been shown to have a protective effect on mitochondrial ultrastructure under these conditions (Vartapetian *et al.*, 2003), but recent evidence suggests that nitrite confers similar protection, and that the reduction of nitrite to NO under hypoxia is important for the maintenance of some level of mitochondrial activity (Gupta *et al.*, 2017). Thus incubating hypoxic pea root mitochondria with 0.5 mM nitrite resulted in increased NO production, improved mitochondrial integrity, improved energization of the inner mitochondrial membrane, increased ATP synthesis, lower levels of reactive

oxygen species, and decreased lipid peroxidation. Nitrite also increased the activities of complex I and the supercomplex I + III_2 under hypoxia. These observations highlight the far-reaching effects of nitrite on the hypoxic mitochondrion (Gupta *et al.*, 2017).

The effect of nitrite on the activities of complex I and the supercomplex I + III_2 under hypoxia (Gupta *et al.*, 2017) may well be important in promoting the reduction of nitrite to NO through the maintenance of a fully functional electron transport chain. In tobacco plants deficient in complex I, reduced electron flow in the mitochondrial ETC led to lower NO production (Shah *et al.*, 2013); while supercomplex formation is considered to increase the efficiency of electron transport (Cogliati *et al.*, 2016). The fact that nitrite treatment under hypoxia increases the activities of complex I and the supercomplex I + III_2 hints at a regulatory role for either nitrite or NO under these conditions.

Interpretation of all the effects of nitrite on mitochondrial function under hypoxia is complicated by the potential regulatory effects of the hypoxically-generated NO. For example, COX, the major site for the production of hypoxically-generated NO, is inhibited by NO (Cleeter *et al.*, 1994) and the interaction with NO can increase the efficiency of oxidative phosphorylation (Clerc *et al.*, 2007). These factors are seen in the nitrite-stimulated increase in ATP synthesis in hypoxic pea root mitochondria (Gupta *et al.*, 2017), but at the same time it should be noted that NO scavenging by mitochondria, or via cytosolic scavenging systems, is considerable, with tobacco root mitochondria, for example, consuming 87% of the NO applied within two minutes (Kumari *et al.*, 2016).

Hypoxically-produced NO may also alter mitochondrial activity through changes in AOX activity. There are established links between AOX and NO, with AOX preventing excess NO production in tobacco leaves (Cvetkovska and Vanlerberghe, 2012), and NO inducing AOX under hypoxia (Gupta *et al*., 2012) and phosphate

deficiency (Royo *et al.*, 2015). Recently, Vishwakarma *et al.* (2018) demonstrated that AOX prevents excess production of NO, peroxynitrite and tyrosine nitration under normoxia. However, it was also found that AOX can generate NO under hypoxia, and that the NO was oxidized via the Pgb-NO cycle (Vishwakarma *et al.*, 2018). Inhibiting AOX under hypoxia led to lower ATP, but AOX overexpressing lines produced more ATP. These data suggested that AOX-mediated NO production plays a role in the production of ATP under hypoxia by supporting proton translocation through complex I. Interestingly, in contrast to normoxia, it was shown that excess NO generated under hypoxia did not lead to the formation of peroxynitrite and tyrosine nitration. Thus, the link between AOX and NO differs between normoxia and hypoxia.

The phenomena of nitrite-driven ATP synthesis and mitochondrial protection are important in specialized structures such as nodules (Berger *et al.,* 2018). *Medicago truncatula* nodules have been shown to increase their production of NO when submitted to hypoxic conditions (Horchani *et al.*, 2011). The nodule oxygen concentration in the cytosol of the host plant cells is typically in the range 5-60 nM due to diffusion resistance and the respiration of the bacteroids. Under these conditions AOX does not contribute to respiration due to its higher K_m, but COX with a K_m value of 50 nM (Millar *et al.*, 1995) is expected to be functional. However, whether the amount of oxygen supplied by leghemoglobin (K_m for oxygen binding 2 nM) to the mitochondria is sufficient for energy production remains an open question (Horchani *et al.*, 2011).

As both Lb and Pgb have the capacity to oxidize NO or nitrate, this may allow the Pgb-NO cycle to operate generating a limited amount of ATP to sustain nodule development and function. The study by Horchani *et al.* (2011) provided evidence that in N_2-fixing nodules of *M. truncatula*, the energy status of the nodules depends largely on NR functioning under normoxic, or hypoxic conditions. Thus, the Pgb-NO cycle can increase energy efficiency in specialised hypoxic organs such as nodules.

Role of NO in oxygen sensing under hypoxia

The precision and specificity of the control of molecular and physiological responses to low oxygen stress (Geigenberger *et al.*, 2000) suggests that plants possess sensitive oxygen-sensing mechanisms to initiate hypoxic responses. Direct and indirect sensors help in the development of these responses. Direct sensors are specific proteins such as transcriptional activators or repressors that sense oxygen. Prominent examples are the transcription factor hypoxia-inducible factor-1-alpha (HIF-1α) in animals (Brahimi-Horn *et al.,* 2005), and a heme-binding protein kinase, FixL in rhizobial bacteria (Akimoto et al., 2003). In contrast, indirect sensing relies on hypoxically-induced changes in such properties as calcium levels, energy status and redox status to trigger regulatory mechanisms (Bailey-Serres and Chang, 2005).

Recently, the direct oxygen sensing mechanism known as the N-end pathway has been shown to initiate the response of plants to hypoxia (Licausi *et al.,* 2011; Gibbs *et al.,* 2011). This is an evolutionarily conserved pathway for protein degradation whereby the stability of a protein is determined by the identity of its N-terminal residues (Varshavsky, 2011; Gibbs *et al.* 2014a; 2015). Specifically, the presence of N-degrons and N-terminal destabilizing residues determines whether a protein will be degraded by the proteasome (Graciet *et al.*, 2009; Holman *et al.*, 2009). Group VII ethylene response factors (ERFs), which have been shown to be important regulators of the response to low oxygen (Hinz *et al.*, 2010; Licausi *et al.*, 2010, 2011; Gibbs *et al.*, 2011*,* 2015), are oxygen-dependent substrates of the N-end rule pathway: these proteins are destabilised in the presence of oxygen and NO and stabilised in their absence. More recently, the polycomb repressive complex 2 component VRN2 has also been identified as an O_2/NO regulated target of the N-end rule pathway, suggesting a potential link between low oxygen/NO and the epigenetic control of gene expression (Gibbs *et al.,* 2018).

The N-terminal (Nt) MCGGAII/L domain of the ERFVII transcription factors is the target for the N-end rule degradation pathway. Under aerobic conditions, methionine amino peptidase cleaves the Nt-Met to reveal an Nt-Cys, which is then oxidized by

plant cysteine oxidases (PCOs) to produce Nt-Cys sulfinate. The oxidized Nt-Cys is arginylated by an arginyl transferase, creating a substrate for an E3 ligase which leads to polyubiquitination and proteasomal degradation of the ERFVII protein (Figure 2). The role of the PCOs in controlling hypoxic gene expression has been confirmed by genetic studies (Weits *et al.*, 2014) and the molecular mechanism of the oxidation and arginylation steps has been characterized *in vitro* (White *et al.*, 2017).

The extent to which NO influences the oxygen sensing system in plants is unclear. In animal systems, it has been shown that the *in vivo* oxidation of Nt-Cys before arginylation requires NO (Hu et al., 2005) and the hydrolysis of *S*-nitrosothiols can produce sulfenic acids as the first step in the formation of sulfinic acids (Reddie and Carroll, 2008). In agreement with this, there is some evidence that NO, as well as oxygen, may be required for the degradation of ERFVIIs. Gibbs *et al.* (2014b) showed that ERFVIIs are destabilized in the presence of NO, and stabilized in their absence. In particular, the stability of two ERFVIIs, RAP2.3 and HRE2, was increased in Arabidopsis seedlings in the presence of NO scavengers, and also in in the nitrate reductase-deficient *nia1nia2* mutant, which has greatly reduced levels of NO. More recently, Vicente *et al.* (2017) demonstrated that down-regulation of nitrate reductase in Arabidopsis led to lower NO levels and increased stability of ERFVIIs, an effect which was implicated in abiotic stress sensing under normoxia. While it remains the case that low oxygen is the primary determinant of ERFVII stability, the sensitivity of ERVIIs to NO raises the possibility that the change in NO levels under hypoxia could modulate the oxygen sensing role of the ERFVIIs.

Another consideration, which needs further investigation, is the potential impact of ROS generation under low oxygen (Vergara *et al.*, 2012). In principle, this could oxidise the Cys residues of ERFVIIs and thus work against their stabilization under hypoxia. At the same time, hypoxia-induced NO generation by mitochondria could play a role in removing excess ROS to ensure the stability of ERFVIIs under hypoxia, but the extent to which this is important has yet to be established and could well

differ between nitrate- and ammonium-grown plants (Wany *et al.*, 2019). More generally the sensitivity of mitochondrial activity to oxygen availability might suggest that mitochondrially-derived NO and ROS could contribute to retrograde signalling in the hypoxic state, but this also remains to be established.

Concluding remarks

It is now clear that nitrite and NO play important and multi-faceted roles in the response of plants to hypoxia. These include classical morphological changes such as hyponasty, the protection of mitochondrial structure, ATP generation and ROS scavenging. However, the role of hypoxically produced NO in various other plant anatomical adaptive responses to flooding, such as aerial lateral root formation, stem elongation, suberin and lignin accumulation needs more investigation. Cross talk between NO and growth hormones such as ethylene, auxin, and ABA during these adaptive responses to hypoxia would also merit further investigation. Another area of interest is the potential effect of the nitrogen supply on hypoxic tolerance given that both nitrite and NO are derived from nitrate. For example, lines with differing nitrogen use efficiency could improve the availability of nitrate and hence affect tolerance to hypoxia. Other targets for further analysis of the role of nitrite and NO under hypoxia include germinating seeds, which experience varying degrees of hypoxia during development, and bulky tissues, where the availability of nitrite might be key to the maintenance of metabolism through its protective effect on the mitochondria. Finally, soil microbes can produce high levels of NO during hypoxia which raises the important question of whether plants are able to distinguish soil-derived NO from that produced endogenously.

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Figure Legends:

Figure 1: Operation of the phytoglobin/nitric oxide (Pgb/NO) cycle under hypoxic conditions. The reduction of nitrite to NO occurs at complex III (bc_1), complex IV (cytochrome *c* oxidase), and the alternative oxidase (AOX). The NO diffuses to the cytosol where it is converted to nitrate (NO_3^-) by the hypoxia-induced class 1 phytoglobin ($PgbO_2$), which leads to the formation of metphytoglobin (MetPgb), which is reduced by metphytoglobin reductase (MetPgbR). Nitrate is then reduced by nitrate reductase (NR) to nitrite, which is imported into mitochondria by either a putative nitrite transporter (NT) or passive diffusion. NAD(P)H generated in the cytosol is oxidized by the externally facing calcium-dependent mitochondrial dehydrogenases (ND), or, after import into the mitochondria as reducing equivalents, by complex I. Cyt *c*, cytochrome *c*; IMM, inner mitochondrial membrane; IMS, intermembrane space; UQ, ubiquinone.

Figure 2: A. The role of nitric oxide (NO) and oxygen in the control of group VII ethylene response factor (ERF) stability under normoxia. Methionine amino peptidase (MetAP) cleaves the Nt-Met to reveal an Nt-Cys, which is then oxidized by plant cysteine oxidases (PCOs) to produce Nt-Cys sulfinate (shaded yellow symbol). NO potentially facilitates this oxidation. The oxidized Nt-Cys is arginylated by an arginyl-tRNA protein transferase (ATE1/2), creating a substrate for the N-end rule E3 ligase (PRT6) which leads to polyubiquitination and proteasomal degradation of ERFVII. It has been shown that NO is also required for degradation of ERFVIIs, and that stress induced reductions in endogenous NO levels can lead to enhanced stability of ERFVIIs even under normoxia (Vicente *et al.,* 2017), thus identifying NO as a signal controlling the accumulation of these proteins in response to stress.

B. Under hypoxia, high levels of NO are produced via increased activity of nitrate reductase and mitochondrial nitrite reduction. However, because ERFVII degradation also requires oxygen, these increased levels of NO are not able to degrade ERFVIIs. The NO generated under hypoxia can help in plant survival via aerenchyma formation, protection of mitochondria, hyponasty, regulation of reactive oxygen species (ROS), the Pgb-NO cycle, and the limited production of ATP. Some of these

adaptive responses are mediated by NO, and some are mediated by stabilization of ERFVIIs or both.

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Fig 1